ELSEVIER

European Journal of Pharmaceutics and Biopharmaceutics 51 (2001) 119–125

European Journal of Pharmaceutics and Biopharmaceutics

www.elsevier.com/locate/ejphabio

Research paper

Reconstruction of an in vitro cornea and its use for drug permeation studies from different formulations containing pilocarpine hydrochloride

Stefanie Tegtmeyer, Ioannis Papantoniou, Christel C. Müller-Goymann*

Institut für Pharmazeutische Technologie, TU Braunschweig, Braunschweig, Germany

Received 25 April 2000; accepted in revised form 25 September 2000

Abstract

The aim of the present contribution was to develop a functional three-dimensional tissue construct to study ocular permeation of pilocarpine hydrochloride from different formulations. The in vitro model was compared to excised bovine cornea. Modified Franz cells were used to study the transcorneal permeability. Analysis was performed by reversed-phase high-performance liquid chromatography. Comparisons of the permeation rates through excised bovine cornea and the in vitro model show the same rank order for the different formulations. The permeation coefficient, K_P, obtained with the cornea construct, is about 2–4-fold higher than that from excised bovine cornea. It is possible to reconstruct bovine cornea as an organotypic culture and also to use this construct as a substitute for excised bovine cornea in drug permeation studies in vitro.

Keywords: Cornea; Cell culture; Permeation study; In vitro model; Pilocarpine hydrochloride

1. Introduction

Investigation of the delivery of drugs to the eye is an indispensable prerequisite for the rational development of ophthalmic dosage forms. Drug delivery to the eye is usually studied in vitro by using either the whole eye or the excised cornea of slaughtered animals [1,2]. Besides high costs for laboratory animals, experiments are costly and time-consuming. Also, interindividual variations in permeability exist, even within the same species. Cell culture models are becoming more established as an alternative to experiments using animals. This approach requires the development of a functional three-dimensional tissue construct [3]. An advantage of such cornea constructs is the absence of interindividual variations observed when using the excised cornea. The problem, however, is to obtain an in vitro cornea with similar barrier properties for the respective drugs as the excised cornea.

The aim of the present contribution was to study the transcorneal permeability of a drug from different formulations for both an organotypic cornea construct and the excised bovine cornea. For this purpose a model of an in vitro cornea from an organotypic culture composed of bovine corneal epithelial cells cultivated on keratocytes in a collagen matrix with an underlying layer of bovine endothelial cells had to be developed [4,5]. All three different cell types were first isolated in primary culture [6].

Then, the organotypic construct, as well as the excised bovine cornea, were tested for their drug permeability using the same formulations including aqueous, oily and a reversed micellar solution of pilocarpine or pilocarpine hydrochloride. The latter formulation was designed to enable sustained drug release on contact with tears via application-induced transformation into a lamellar liquid crystal [7]. This effect has already been proven to be successful for rectal [8] and intramuscular administration [9].

2. Materials and methods

2.1. Materials

Pilocarpine hydrochloride (PHCl) was supplied by Ciba-Vision AG (Aschaffenburg, Germany); isopropylmyristate (IPM) was provided by Henkel (Düsseldorf, Germany). The lecithin used was Phospholipon 90 G® (Rhone-Poulenc Rorer, Cologne, Germany), which consisted of pure soya lecithin with a content of at least 90% phosphatidylcholine. The applied buffer was an isotonic phosphate buffer, pH 7.0. Borocarpin®-S 2% and Pilocarpol® 2% eyedrops were

* Corresponding author. Institut für Pharmazeutische Technologie, TU Braunschweig, Mendelssohnstrasse 1, D-38106 Braunschweig, Germany. Tel.: +49-531-391-5650; fax: +49-531-391-8108.

E-mail address: c.mueller-goymann@tu-bs.de (C.C. Müller-Goymann).

0939-6411/01/$ - see front matter
PII: S0939-6411(01)00123-0

supplied by Dr Winzer (Olching, Germany), Spersacarpin® 2% was purchased in a retail pharmacy. Table 1 shows the composition of the different eyedrops.

2.2. Preparation of the reversed micellar solution

While being stirred with a Teflon coated magnet, lecithin was dissolved in IPM at a temperature of 50°C, resulting in a clear, yellowish reverse micellar solution. PHCl was dissolved in water. A dispersion of a lamellar liquid crystal was obtained upon adding the PHCl solution into the reverse micellar solution. The dispersion was stirred at 50°C for 24 h to remove the water and to obtain again a drug-loaded reverse micellar solution.

2.3. Preparation of excised bovine cornea

Eyes from adult domestic cattle were obtained from a local slaughterhouse. The cornea was excised with an attached 1–2-mm wide scleral ring. Connective tissue and external muscles were then removed. The corneas were rinsed with phosphate-buffered saline containing antibiotic solution 1% (prepared with 10 000 units/ml penicillin G sodium, 10 mg/ml streptomycin sulfate and 25 μg/ml amphotericin B as Fungizone® in 0.85% saline) (Gibco, Munich, Germany).

2.4. Primary cultures and subcultivation

To obtain endothelial cells the cornea was placed, endothelial side up, into a high-grade steel cup, having the same shape as the cornea [10]. The endothelium was covered with trypsin–EDTA solution (Gibco) and incubated for 7–8 min. The cells were dislodged with a rubber spatula to avoid damaging the descemet membrane and contamination with keratocytes from the underlying stroma), and cultivated in a 35-mm diameter dish (Costar, Fernwald, Germany) [11,12].

To obtain bovine stromal corneal cells, both endothelial and epithelial sheets were removed and 4-mm explants attached to a 35-mm diameter dish. Growth of stromal corneal cells occurred after 6–7 days.

Tissue culture of the corneal epithelial cells is a well-established technique [13,14], used in many laboratories for studying a variety of factors [15]. Bovine corneal epithelial cells were derived from explants which were placed stromal side down (endothelium was removed) in a 35-mm diameter dish. The explants were allowed to adhere to the surface of the well for 1 h at 37°C in a humid 5% CO_2 + 95% air atmosphere. Growth medium, Dulbecco's modified Eagle's medium (DMEM) (ICN, Eschwege, Germany) supplemented with 10% newborn calf serum (NBCS) (Life Technologies, Eggenstein, Germany), was added to cover the explants without dislodging them. Cellular growth was observed after 3 days. In order to prevent contamination by keratocytes the explants were removed with sterile forceps after 4 days.

All three cell types were cultivated in DMEM supplemented with NBCS 10%, 4 mM L-glutamine and antibiotic solution 1% (Gibco). Growth medium was changed three times a week. All subcultures were passaged until the 20th generation and maintained their characteristic morphologies without showing any signs of senescence.

After the first passage the cell yield for the endothelial cornea cells was $4.8 \pm 1.2 \times 10^4$/ml and for epithelial cells $5.8 \pm 1.8 \times 10^4$/ml. Stromal corneal cells show the highest growth rate and yielded $6.9 \pm 3.1 \times 10^4$/ml. This cell amount provides at least a fivefold cell number after the next passage.

2.5. Characterization of cell types

The monoclonal antibody anti-epithelial keratin AE5 (Sigma, Deideshofen, Germany) was used to identify keratin in corneal epithelial cells. The monoclonal antibody anti-vimentin clone V9 (Sigma) was used to identify vimentin in endothelial and stromal corneal cells. All cell types were first incubated for 30 min in a moist chamber with the described antibodies, diluted 1:100. The slides were then rinsed with PBS. A peroxidase conjugated anti-mouse IgG antibody (Sigma) was used as secondary antibody, diluted by 1:100 and incubated for 30 min in a moist chamber. After rinsing with PBS, the addition of diaminobenzidine (0.5 mg/ml)/hydrogen peroxide (0.3 mg/ml) to the conjugated peroxidase led to the formation of a brown stain. The cells were examined by light microscopy.

Stromal corneal cells were differentiated from endothelial cells microscopically.

2.6. Corneal tissue construct

Bovine endothelial cells ($3–4 \times 10^4$/ml) were seeded onto a polycarbonate filter Transwell culture insert (Costar, Fernwald, Germany) of an underlying layer of type I collagen gel lattice and grown for 1 week to confluence. Pure collagen (type I) was extracted from rat tails according to the method

Table 1
Composition of the different eyedrops

	Active substance (1 g contains:)	Other ingredients (1g contains:)
Borocarpin-S 2%	20 mg pilocarpine hydrochloride in an aqueous solution	0.1 mg benzalkonium chloride
Spersacarpin 2%	20 mg pilocarpine hydrochloride in an aqueous solution	0.1 mg benzalkonium chloride 4.5 mg hydroxypropyl methylcellulose
Pilocarpol 2%	20 mg pilocarpine in an oily solution	0.002 mg cetalkonium chloride

of Bell et al. [16]. A collagen layer containing 5×10^4/ml bovine stromal corneal cells [17] was then cast atop the confluent endothelial cell layer and allowed to gel at room temperature after neutralization with $NaHCO_3$. The gel was allowed to contract submerged in DMEM–10% NBCS for about 7 days in a humidified incubator at 37°C and 5% CO_2. When gel contraction was finished, bovine epithelial cells in a concentration of $2–3 \times 10^5$/ml were seeded onto the central area of the contracted lattice and grown submerged in a 3:1 mixing of glucose-free DMEM (ICN, Eschwege, Germany) and Ham's F12 supplemented with newborn calf serum (20 μl/ml), insulin (5 μg/ml), triiodothyronine (20 pM), transferrin (5 μg/ml), adenine (24.3 μg/ml) (Sigma), ethanolamine (6.1 μg/ml), phosphoethanolamine (14.1 μg/ml), selenious acid (6.8 ng/ml) (Biochrom, Berlin, Germany), penicillin G sodium (100 units/ml), streptomycin sulfate (100 μg/ml) and amphotericin B (0.25 μg/ml) (Gibco). After approximately 7 days the cultures were lifted to the air–liquid interface for an additional 2 weeks, while the medium was lowered concurrently to just meet the surface of the culture. A schematic representation of the development is shown in Fig. 1.

2.7. Light microscopy

For histology, corneal tissue constructs were embedded in paraffin after fixation in 8% formalin and dehydrated in a graded ethanol series. Four-micrometer cross sections were cut, stained in hematoxylin–eosin, examined and photographed using a photomicroscope (Zeiss, Oberkochen, Germany).

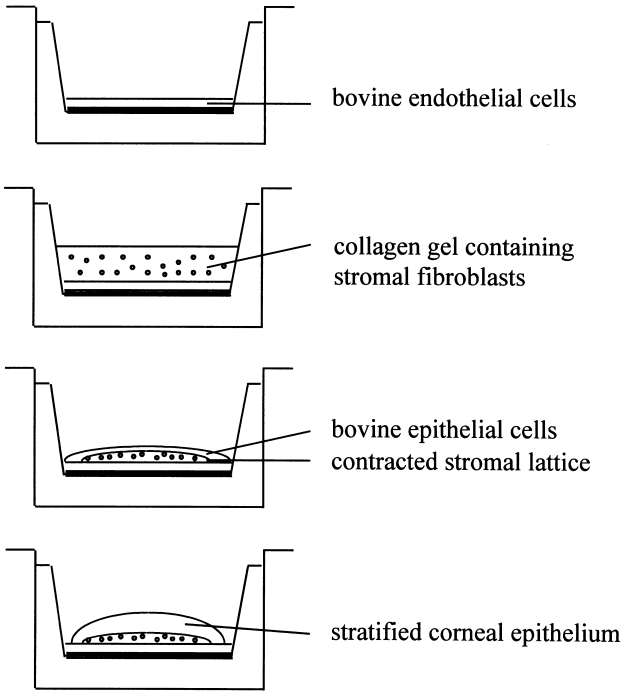

Fig. 1. Schematic representation of the development of the corneal tissue construct.

2.8. Permeation studies

For the comparative evaluation of the barrier function, i.e. the transcorneal drug permeability, both the excised bovine cornea and the cornea construct were mounted into a modified Franz cell. For stabilization, the organotypic culture remained on the 3-μm pore polycarbonate filters from the Transwell culture insert, used for culture. Due to the wide pore size drug permeability was not modified at all by the filter. Permeation studies were performed at least in triplicate. The permeability coefficients K_P were calculated as flux/drug concentration from the linear slopes of the permeation curves. The acceptor solution was an isotonic phosphate-buffered saline, pH 7.0, and the volume was 6.2–6.5 ml, depending on the particular Franz cell. The donors were Borocarpin-S 2% which is an aqueous solution of pilocarpine hydrochloride, Spersacarpin 2% which contains, in addition, hydroxypropyl methylcellulose in an aqueous solution for an increased viscosity, and Pilocarpol 2% as an oily solution of pilocarpine and the reverse micellar solution, described above. The volume of the donor was always 660 μl.

Pilocarpine analysis was performed by reversed-phase high-performance liquid chromatography using a Gromsil ODS 5 μm, 125 × 4 mm (Grom, Herrenberg, Germany) [7]. The mobile phase consisted of acetonitrile 77 parts and buffer 23 parts, pH 7.0 (6.8 g/l KH_2PO_4, 1.7g/l KOH, 3.6 g/l NaCl). A flow rate of 1.0 ml/min using a Spectroflow 400 pump (Kratos, Weiterstadt, Germany) was used. Peaks were detected with a Spectroflow 757 absorbance detector (Kratos) at 215 nm. Data analysis and calculation were performed by Beckman System Gold Chromatography software version 6.01 (Beckman, Munich, Germany). Calibration was carried out with six different concentrations within a range of 0.2–25 μg/ml with a correlation coefficient of 0.99997.

3. Results and discussion

Successful cultivation of an organotypic three-dimensional tissue construct from pure cultures of corneal endothelial, stromal and epithelial cells from bovine cornea was carried out. Corneas of 30 slaughtered cattle were used

Table 2
Characterization of different corneal cell types

	Epithelial cells	Stromal cells	Endothelial cells
Keratin-positive	+	−	−
Vimentin-positive	−	+	+
Microscopical appearance	Polygonal shape	Elongated spindle shape	Polygonal shape

as the starting material for establishing primary cultures and subculturing. Amongst the variable material no considerable difference was observed. Furthermore, the cultured cells were characterized by immunocytochemical methods and with respect to morphological appearance and cell size until the 20th generation without showing signs of senescence. The corneal epithelial and endothelial cells show a polygonal shape. Stromal corneal cells are elongated, having a typical spindle shape. The results are summarized in Table 2.

Only passages four to eight were used for the reconstruction of the in vitro cornea. With these higher passages the enormous increase in cell number enables the reconstruction of more than 100 artificial corneas from one excised bovine cornea.

Fig. 2A shows a cross section of the epithelium with

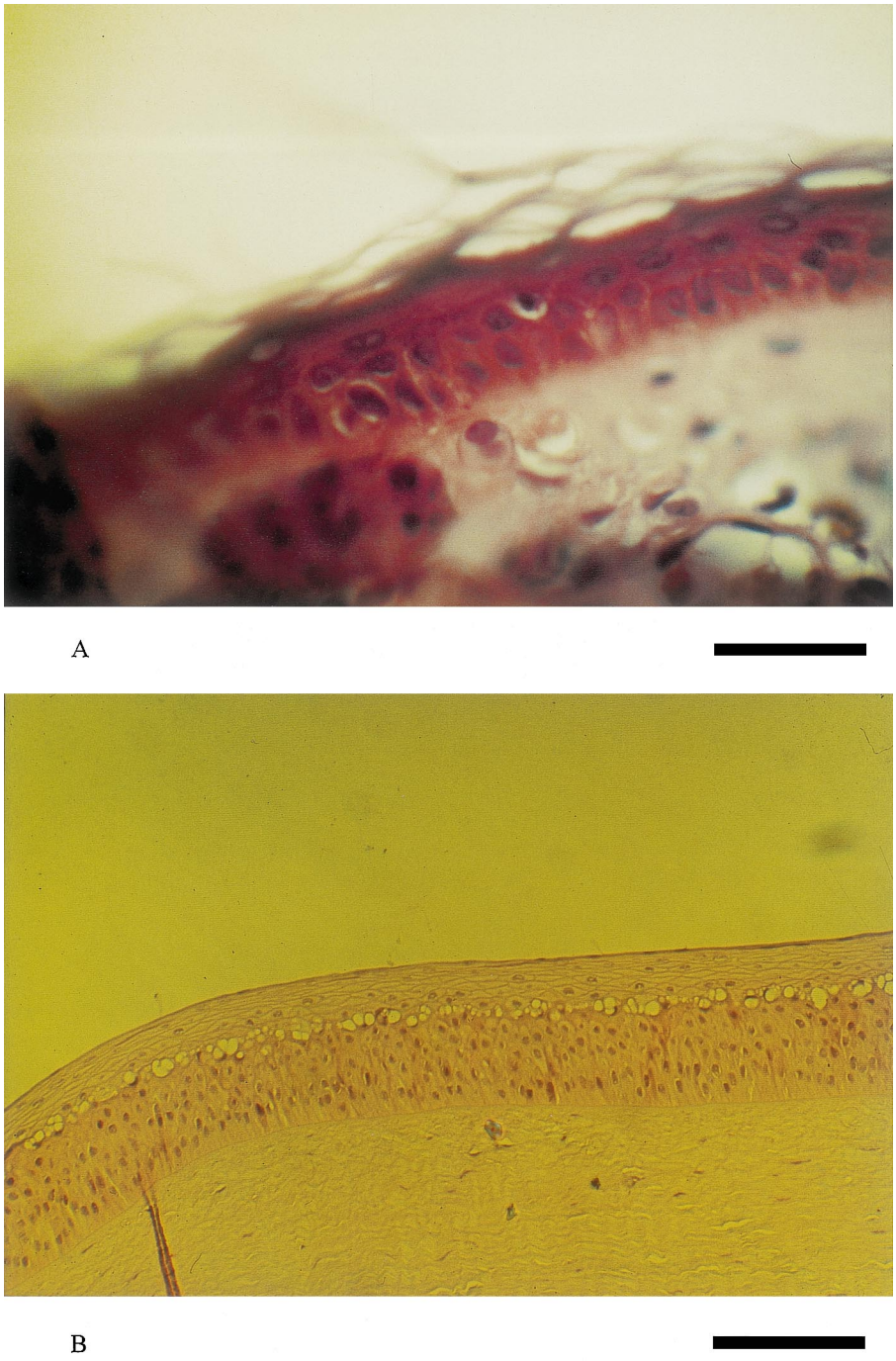

Fig. 2. (A) Cross section of the epithelium region with underlying stroma of a corneal tissue construct. Bar: 15 μm. (B) Cross section of the epithelium region with underlying stroma of an excised bovine cornea. Bar: 60 μm.

Table 3
Comparison of the permeability coefficients K_P (cm/s) of PHCl and pilocarpine from Borocarpin-S, Spersacarpin, Pilocarpol and the reverse micellar solution (RMS)

	Excised bovine cornea (mean ± SD)	SD_{rel} (%)[a]	Organotypic culture (mean ± SD)	SD_{rel} (%)
Borocarpin-S	$2.29 \times 10^{-6} \pm 0.73 \times 10^{-6}$	31.9	$8.59 \times 10^{-6} \pm 1.38 \times 10^{-6}$	16.1
Pilocarpol	$1.14 \times 10^{-6} \pm 0.17 \times 10^{-6}$	14.9	$2.51 \times 10^{-6} \pm 0.39 \times 10^{-6}$	15.5
Spersacarpin	$7.77 \times 10^{-7} \pm 1.57 \times 10^{-7}$	20.2	$13.37 \times 10^{-7} \pm 0.74 \times 10^{-7}$	5.5
RMS	$6.11 \times 10^{-8} \pm 1.15 \times 10^{-8}$	18.8	$2.54 \times 10^{-7} \pm 0.39 \times 10^{-7}$	15.4

[a] SD_{rel}, relative standard deviation.

underlying stroma of an organotypic tissue culture of bovine corneal cells cultivated 2 weeks at the air–liquid interface. Light microscopic analysis indicated that cultured corneal epithelial cells on the collagen gel lattice with incorporated keratocytes had grown to 4–5 layers. A basal cell layer is detectable, together with two layers of suprabasal cells and a layer of stratified, flattened cells on top. The epithelium reveals a similar morphology to the epithelium in the excised cornea. Fig. 2B shows a cross section of the epithelial region with underlying stroma of the excised bovine cornea.

To investigate whether the morphological similarities between the construct and the excised cornea also result in a similar barrier function, the permeation of different model formulations of PHCl was investigated using a modified Franz cell. The permeation coefficients (K_P) of PHCl for the various formulations are shown in Table 3.

The results from the excised cornea studies are in accordance with those obtained by Suhonen et al. [18] and Siefert et al. [19]. The various formulations show significantly different profiles for the permeation across excised bovine cornea (Fig. 3). The aqueous solution of PHCl, without thickening additives, has the highest permeation coefficient. A reverse micellar solution of PHCl, which transforms into a lamellar liquid crystal on contact with tear fluid upon application, exhibits a permeation coefficient which is reduced by a factor of 37. The permeation profiles of the other two formulations lie between these extremes. The differences in K_P are likely to be results of the described different compositions of the formulations, but not of a changed permeability of the cornea.

Determination of the permeation of the same formulations through the organotypic three-dimensional model system of bovine cells leads to the same rank order of the different formulations in terms of K_P (Figs. 3 and 4, Table 3). The permeation coefficient K_P is just 2–4-fold higher with the cornea construct than with the excised bovine cornea. In comparison with similar permeation experiments across organotypic cultures of human dermal and epidermal cells and the excised human stratum corneum [20], a smaller difference between the permeation coefficients of the in vitro model and the original excised barrier is obtained. Therefore, it can be concluded that the barrier function of the cornea construct for a drug such as pilocarpine hydrochloride is very similar to that of the excised bovine cornea. Studying the barrier function of the cornea construct with an ophthalmologically applied hydrophilic paracellular permeant, like mannitol or carboxy fluorescein, offers no rational permeability data, because pilocarpine hydrochloride permeates through the corneal epithelium mostly transcellularly, whereas hydrophilic permeants give only information about the tightness of the intercellular space.

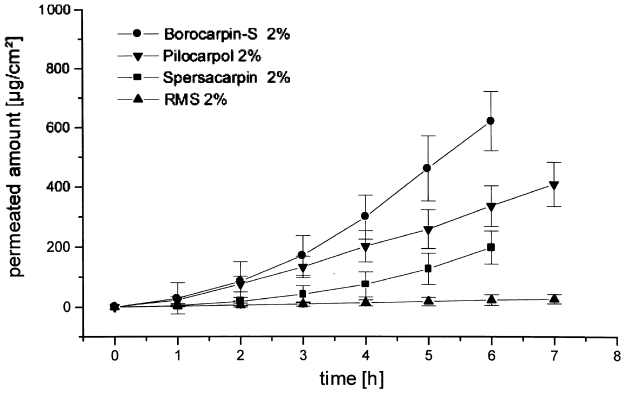

Fig. 3. Permeation of PHCl from Borocarpin-S ($n = 8$), Spersacarpin ($n = 6$), the reverse micellar solution ($n = 7$) and of pilocarpine from Pilocarpol ($n = 6$) across excised bovine cornea. Graphs represent mean and standard deviation.

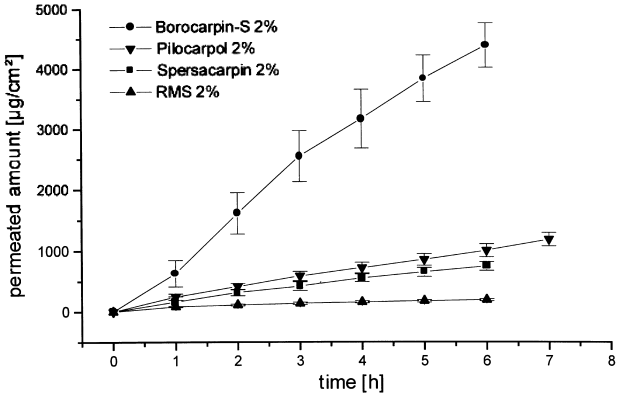

Fig. 4. Permeation of PHCl from Borocarpin-S ($n = 3$), Spersacarpin ($n = 3$), the reverse micellar solution ($n = 4$) and of pilocarpine from Pilocarpol ($n = 5$) across organotypic cultures. Graphs represent mean and standard deviation.

The appropriateness of using the construct instead of the original cornea is further demonstrated by the same rank order of the drug permeabilities from the different formulations in both the construct and the excised bovine cornea. Furthermore, apart from one exception, the relative standard deviation of the permeation coefficient with the in vitro cornea is smaller than that with the excised bovine cornea. The permeation of pilocarpine hydrochloride from Borocarpin-S is about 34–37 times faster in both models than that from the reverse micellar solution (i.e. of the lamellar liquid crystal) across the investigated permeation barriers. Spersacarpin shows a slight sustained release in both experiments which might depend on the amount of hydroxypropyl methylcellulose [21] and therefore the higher viscosity of the solution. Pilocarpol is an oily preparation which hinders vision [21], and is consequently used for administration in the evening to prevent high intraocular pressure overnight. For the latter kind of administration a greater sustained effect is even more desirable. The present investigation demonstrates that a reversed micellar solution may be useful for this purpose, provided that both a higher drug load and a higher release rate can be achieved.

A general disadvantage of using in vitro permeation studies, however, is that an in vitro model does not take into account the composition of aqueous humor, the composition of tear fluid, the mechanical stress of the eyelids and tear flow.

4. Conclusion

A functional in vitro model of a cornea has been developed, which may be useful for ophthalmic permeation studies. Although drug permeability via these organotypic cultures is increased by factor of 2–4 in comparison with excised bovine cornea, permeation studies with these cornea constructs enable the determination of variations in ocular drug availability from different formulations. Further studies with other drugs will have to show whether the factor of 2–4 will hold in general.

It can be anticipated that constructs based on human corneal cell may also be developed. Constructs based on human cells would be an ideal barrier for the in vitro investigation of drug permeation, as excised human cornea is not available for this purpose.

Furthermore, the investigation shows that a reverse micellar solution, which exhibits an application-induced transformation into a semisolid system of liquid crystalline microstructure on contact with tears, may be appropriate for sustained drug release overnight.

Acknowledgements

We wish to thank Dr E. Parussis, Braunschweig, for preparing the cross sections of the cultures, Dr Lücking, Hannover, for the supply of bovine eyes, and Rhone-Poulenc Rorer, Ciba-Vision and Dr Winzer for the supply of materials.

References

[1] Y.P. Zhu, C.G. Wilson, W.S. Wilson, An ex vivo model for the assessment of drug delivery to the eye: isolated bovine eye perfusion system, Eur. J. Pharm. Biopharm. 42 (1996) 405–410.
[2] J.B. Richman, D.D.S. Tang-Liu, A corneal perfusion device for estimating ocular bioavailability in vitro, J. Pharm. Sci. 79 (1990) 153–157.
[3] Y. Minami, H. Sugihara, S. Oono, Reconstruction of cornea in three-dimensional collagen gel matrix culture, Invest. Ophthalmol. Vis. Sci. 34 (1990) 2316–2324.
[4] J.D. Zieske, V.S. Mason, M.E. Wasson, S.F. Meunier, C.J.M. Nolte, N. Fukai, B.R. Olsen, N.L. Parenteau, Basement membrane assembly and differentiation of cultured corneal cells: importance of culture environment and endothelial interaction, Exp. Cell Res. 214 (1994) 621–633.
[5] A.I. Schneider, K. Maier-Reif, T. Graeve, The use of an in vitro cornea for predicting ocular toxicity, In vitro Toxicol. 10 (1997) 309–318.
[6] F.W. Stocker, A. Eiring, R. Georgiade, N. Georgiade, A tissue culture technique for growing corneal epithelial, stromal, and endothelial tissues separately, Am. J. Ophthalmol. 46 (1958) 294–298.
[7] I. Papantoniou, Invers mizellare Lösungen mit modifizierter Wirkstoffreigabe durch applikationsinduzierte Transformation in lyotrope Mesophasen. Thesis, TU Braunschweig, Braunschweig, 1995.
[8] A. Schneeweis, C.C. Müller-Goymann, In vivo and in vitro diclofenac sodium evaluation after rectal application of soft gelatine capsules enabling application induced transformation (AIT) into a semisolid system of liquid crystals (SSLC) for controlled release, Pharm. Res. 14 (1997) 1726–1729.
[9] A.K. Uppadhyay, L.K. Omray, A.J. Khopade, N.J. Jain, Studies on a reverse micelle-lamellar phase transition based depot preparation of rifampicin, Pharmazie 52 (1997) 961–962.
[10] D.K. MacCallum, J.H. Lillie, L.J. Scaletta, J.C. Occhino, W.G. Frederick, S.R. Ledbetter, Bovine corneal endothelium in vitro, Exp. Cell Res. 139 (1982) 1–13.
[11] J.L. Baum, R. Niedra, C. Davis, B.Y.J.T. Yue, Mass culture of human corneal endothelial cells, Arch. Ophthalmol. 97 (1979) 1136–1140.
[12] K.J. Engelmann, M. Böhnke, P. Friedl, Isolation and long-term cultivation of human corneal endothelial cells, Invest. Ophthalmol, Vis. Sci. 29 (1988) 1656–1662.
[13] B. Ebato, J. Friend, R.A. Thoft, Comparison of central and peripheral human corneal epithelium in tissue culture, Invest. Ophthalmol. Vis. Sci. 28 (1987) 1450–1456.
[14] C.R. Kahn, E. Young, I.H. Lee, J.S. Rhim, Human corneal epithelial primary cultures and cell lines with extended life span: in vitro model for ocular studies, Invest. Ophthalmol. Vis. Sci. 34 (1993) 3429–3441.
[15] P.P. Parnigotto, V. Bassani, F. Montesi, M.T. Conconi, Bovine corneal stroma and epithelium reconstructed in vitro: characterization and response to surfactants, Eye 12 (1998) 304–310.
[16] E. Bell, H.P. Ehrlich, D.J. Buttle, T. Nakatsuji, Living tissue formed in vitro and accepted as skin-equivalent tissue of full thickness, Science 211 (1981) 1052–1054.
[17] E. Bell, S. Sher, B. Hull, C. Merrill, S. Rosen, A. Chamson, D. Asselineau, D. Dubertret, B. Coulomb, C. Lapiere, B. Nusgens, Y. Neveux, The reconstruction of living skin, Invest. Dermatol. 81 (1983) 2s–10s.
[18] P. Suhonen, T. Järvinen, P. Peura, A. Urtti, Permeability of pilocarpic acid diesters across albino rabbit cornea in vitro, Int. J. Pharm. 74 (1991) 221–228.

[19] B. Siefert, S. Keipert, Influence of alpha-cyclodextrin and hydroxyalkylated beta-cyclodextrin derivatives on the in vitro corneal uptake and permeation of aqueous pilocarpine-HCl solutions, J. Pharm. Sci. 86 (1997) 716–720.
[20] C. Specht, I. Stoye, C.C. Müller-Goymann, Comparative investigations to evaluate the use of organotypic cultures of transformed and native dermal and epidermal cells for permeation studies, Eur. J. Pharm. Biopharm. 46 (1998) 273–278.
[21] S. Keipert, Etablierte und neue Konzepte zur Optimierung von Ophthalmika, Pharm. Ztg. 139 (1994) 567–576.